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METHODS PAPER

The role of platelets in the recruitment of leukocytes during vascular disease

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Abstract

Besides their role in the formation of thrombus during haemostasis, it is becoming clear that platelets contribute to a number of other processes within the vasculature. Indeed, the integrated function of the thrombotic and inflammatory systems, which results in platelet-mediated recruitment of leukocytes, is now considered to be of great importance in the propagation, progression and pathogenesis of atherosclerotic disease of the arteries. There are three scenarios by which platelets can interact with leukocytes: (1) during haemostasis, when platelets adhere to and are activated on sub-endothelial matrix proteins exposed by vascular damage and then recruit leukocytes to a growing thrombus. (2) Platelets adhere to and are activated on stimulated endothelial cells and then bridge blood borne leukocytes to the vessel wall and. (3) Adhesion between platelets and leukocytes occurs in the blood leading to formation of heterotypic aggregates prior to contact with endothelial cells. In the following review we will not discuss leukocyte recruitment during haemostasis, as this represents a physiological response to tissue trauma that can progress, at least in its early stages, in the absence of inflammation. Rather we will deal with scenarios 2 and 3, as these pathways of platelet–leukocyte interactions are important during inflammation and in chronic inflammatory diseases such as atherosclerosis. Indeed, these interactions mean that leukocytes possess means of adhesion to the vessel wall under conditions that may not normally be permissive of leukocyte–endothelial cell adhesion, meaning that the disease process may be able to bypass the regulatory pathways which would ordinarily moderate the inflammatory response.

Keywords

Adhesion, inflammation, leukocytes, platelets, vascular disease

History

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Introduction

It is now widely recognised that platelets play an import role in the pathology associated with cardiovascular diseases. The literature on the contribution of platelets to symptomatic atherothrombotic disease, which is predicated on the formation of a mural thrombus in diseased arteries, is extensive. As this aspect of platelet biology is well developed and is already the target of a number of successful and well validated therapeutic pathways, we do not propose to address it in the current discussion. The reader is directed to a recent review of this area for additional information [1].

Here, having briefly revisited endothelial cell (EC) mediated pathways of leukocyte recruitment during inflammation, the roles of platelets in the recruitment and function of leukocytes in cardiovascular diseases will be discussed. In particular, the molecular basis of the interactions between platelets, leukocytes and the vessel wall are reviewed. The ability of immobilised platelets to recruit leukocytes is well established and discussed in detail. However, the developing field of leukocyte–platelet aggregation in circulating blood is also addressed, including emerging paradigms on the importance of platelet microvesicles (PMV) in the regulation of leukocyte recruitment in cardiovascular diseases. Finally, the current therapeutic options for intervening in the platelet-mediated recruitment of leukocytes in cardiovascular diseases will be discussed.

Leukocyte adhesion during resolving inflammation

In vertebrates, infection or tissue trauma cause the rapid initiation of an acute and resolving inflammatory response. The identity of the leukocytes within the tissue infiltrate vary with time and throughout the evolution of the inflammatory response in order that the requirements for repair of the affected tissues and the development of an adaptive immune response are matched to the specialised functions of the different leukocyte subsets [2].

The molecular processes that support leukocyte recruitment in post-capillary venules (the site where leukocytes are recruited during acute inflammation) are well described (Figure 1). Thus, local inflammatory mediators, such as histamine or cytokines (e.g. tumour necrosis factor-α – TNF), activate ECs resulting in

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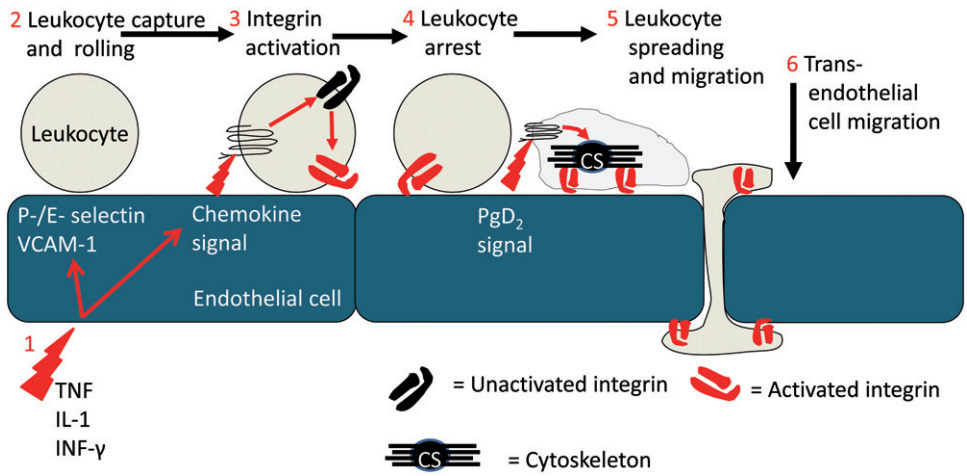

Figure 1. The multi-step leukocyte adhesion cascade: (1) Endothelial cells at a site of inflammation are activated by stromal derived inflammatory cytokines such as TNF-α, IL-1β and IFN-γ. Induction of transcriptional activity results in the expression of adhesion molecules and chemokines which coordinate leukocyte recruitment. (2) Leukocytes are recruited from flowing blood by specialised receptors of the selectin family and VCAM-1, which also support rolling adhesion. (3) Chemokine signals activate the β1 and β2 integrins on rolling leukocytes. (4) Adhesion is stabilised and the leukocytes become firmly adherent to the endothelial cell. (5) In response to additional signals, e.g. from PgD2, the leukocyte cytoskeleton undergoes remodelling driving shape change (spreading) and dynamic integrin-mediated migration. (6) Leukocytes migrate across and through the endothelial cell monolayer and onwards into tissue.

the expression of specialised adhesion receptors that form short lived bonds with counter ligands on the leukocyte surface [3–5]. Thus, E- and P-selectin and the immunoglobulin super-family (IgSF) member, vascular adhesion molecule-1 (VCAM-1), are the major receptors that tether leukocytes from rapidly flowing blood [6]. The sequential formation and dissolution of these bonds supports a characteristic and dynamic form of adhesion, referred to as rolling [7] which does not require leukocyte activation. However, leukocyte migration through the vessel wall and into the inflamed tissue is dependent upon the receipt of an activating stimulus [7, 8]. Peptide agonists of the chemokine family are the major activating stimuli presented to rolling leukocytes in order to stabilise adhesion [9, 10], although numerous other mediators have been reported to play a role in leukocyte recruitment under some inflammatory conditions (e.g. platelet activating factor [PAF] and leukotriene-B4 [LTB4]) [11–14]. These signals result in remodelling of the cytoskeleton and activation of the β1 and β2-integrin adhesion molecules that provide firm adhesion. The integrins also support apical and trans-EC migration of adherent leukocytes using intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 as counter ligands [9]. Efficient onward migration after chemokine activation also requires additional downstream signals. Thus, neutrophils and T-lymphocytes require a prostaglandin-D2 (PGD2) signal to undertake diapedesis *in vitro* [15]. In addition, molecules at the EC junction have been implicated in the regulated trafficking of leukocytes, including CD31 (platelet-endothelial cell adhesion molecule-1 – PECAM-1), CD99 and family members of the junctional adhesion molecules (JAM) family, e.g. JAM-C [16, 17].

Platelets are a sufficient substrate to support the recruitment, activation and migration of blood borne leukocytes

There is now an increasing awareness that aberrant pathways of leukocyte recruitment might be established in disease states [18]. Of relevance here was the increasing realisation that platelets might promote leukocyte adhesion during inflammation, in particular the concept that platelet interactions with the vessel wall might lead to inappropriate leukocyte recruitment in atherosclerosis. Such hypotheses were predicated on the assumption that activated platelets were capable of recruiting leukocytes from the blood. This was a reasonable expectation, as platelet α-granules contain P-selectin which is expressed upon activation [19, 20]. In addition, they release leukocyte activating agents (see below for details).

Early studies showed conclusively that monolayers of immobilised, spontaneously activated platelets were able to recruit flowing leukocytes using P-selectin [21, 22]. Both granulocytic and mononuclear leukocytes were captured efficiently from flow and could roll continuously across the monolayer without apparent signs of activation. Importantly, if the platelets were activated by thrombin (i.e. through protease-activated receptors [PAR] – PAR1 and PAR4), neutrophils were rapidly activated through the CXCR2 receptor, putatively by platelet-derived CXCL7 (NAP-2) [23]. Other studies using activated platelets have identified alternative pathways of neutrophil activation. Thus, thrombin stimulated platelets activate neutrophils through PAF and LTB4 [24], while platelets bound to and activated by collagen type 1, activated neutrophils through PAF [25]. Interestingly, LTB4 is not a platelet-derived eicosanoid, but can be synthesised by neutrophils from platelet-derived arachidonic acid [26], making it an exemplar of transmetabolic activity requiring the integrated activity of the haemostatic and immune systems. Other CXC chemokines have been reported in platelets, although their function in leukocyte recruitment remains undetermined. Thus, a full-length cDNA for CXCL5 (ENA 78) was cloned from human platelets [27], although transcribed protein was not measured. In addition, rabbit platelets contain preformed and stored CXCL8 (IL-8), which was secreted upon platelet activation [28].

Using activated neutrophils, the β2-integrin, CD11b/CD18 (αMβ2; Mac-1) supported immobilisation on the platelet monolayer using platelet fibrinogen as a counter ligand [29, 30]. Interestingly, immobilised platelets could also provide signals through specific receptors (e.g. P-selectin and CD31) that regulated the velocity and direction of neutrophil migration [31, 32]. Moreover, neutrophils migrating on a platelet monolayer formed large embolising aggregates when they came into contact with each other [33]. Diacovo et al. showed that platelets immobilised on fibronectin-coated polycarbonate filters, supported neutrophil migration towards an exogenous chemotactic agent added below the filter [29].

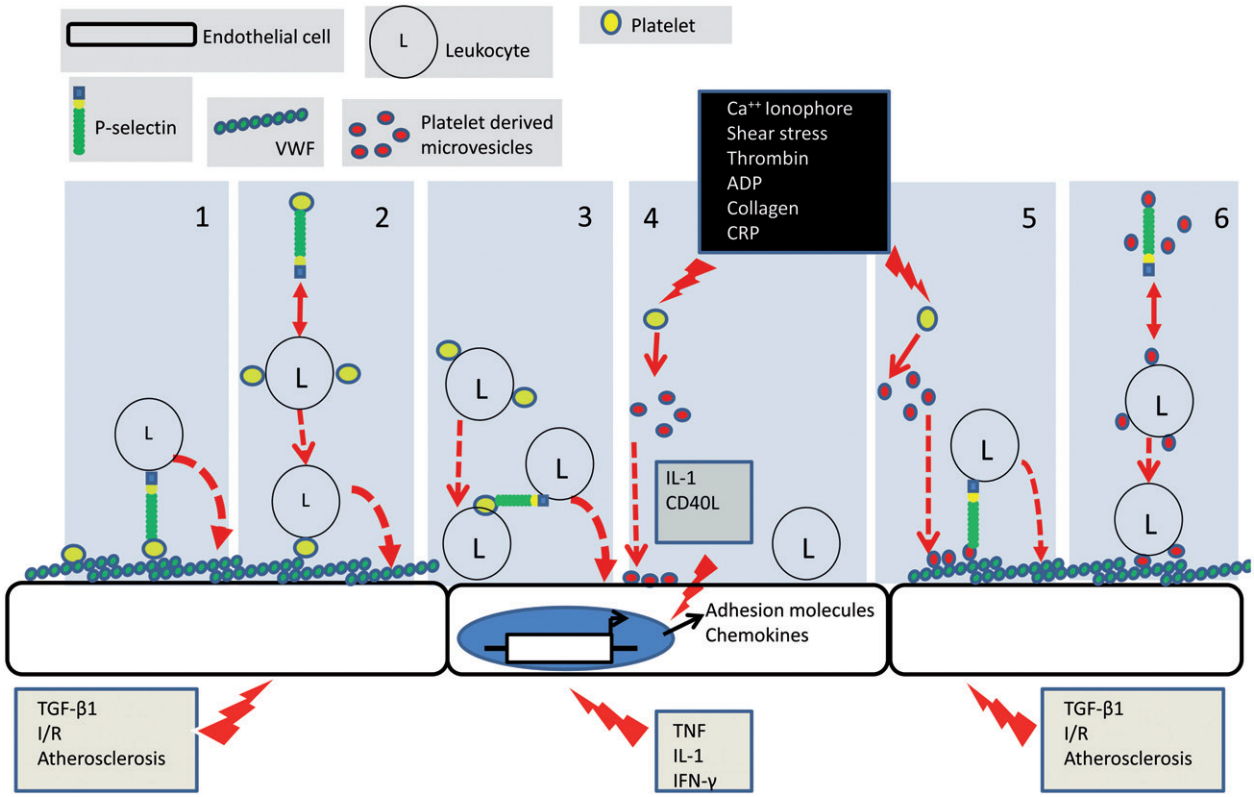

Figure 2. Mechanisms of platelet-mediated leukocyte recruitment: (1) appropriately activated endothelial cells express a matrix of VWF on their surface to which platelets are recruited and activated. Platelet presented P-selectin then forms an adhesive bridge between the endothelial surface and blood borne leukocytes. (2) Platelets form heterotypic aggregates with leukocytes in the circulation in a P-selectin dependent manner. Aggregates are then recruited to the VWF on the surface of appropriately activated endothelial cells. (3) Inflammatory cytokines induce transcriptional programmes in endothelial cells which result in the expression of adhesion molecules and chemokines that support leukocyte adhesion. Leukocytes in heterotypic aggregates which are recruited to the endothelial cell surface posses additional platelet borne receptors. These can in turn support the secondary adhesion of un-aggregated leukocytes. (4) Upon activation platelets shed microvesicles which bind directly to endothelial cells. Microvesicle borne inflammatory cytokines induce transcriptional programmes in endothelial cells which result in the expression of adhesion molecules and chemokines that support leukocyte adhesion. (5) P-selectin bearing PMV bind to appropriately activated endothelial cells. Platelet presented P-selectin then forms an adhesive bridge between the endothelial surface and blood borne leukocytes. (6) P-selectin bearing PMV form heterotypic aggregates with leukocytes in the circulation. Aggregates are then recruited to VWF on appropriately stimulated endothelial cells utilising microvesicle borne adhesion receptors.

Platelets can bind to appropriately stimulated endothelial cell monolayers

The concept that platelets may interact directly with ECs conditioned by an inflammatory environment has received increasing attention. Ordinarily, ECs present an anti-thrombotic surface to flowing blood by releasing nitric oxide (NO) and prostacyclin (PGI2) and through expression of CD39 [34]. Signalling into the platelet by PGI2 and NO maintains high levels of cyclic nucleotides within the platelet cytoplasm, thereby antagonising all the known pathways of platelet activation [35–37], while the ectonucleotidase activity of CD39 enzymatically degrades ADP, one of the two major feedback agonists involved in platelet activation. Early work thus assumed that EC 'damage' was required to expose matrix to which platelets could bind [38, 39]. This paradigm has been modelled *in vitro* using sub-confluent ECs, where matrix proteins readily recruit platelets from perfused blood [40]. However, more recent *in vitro* studies show that platelets can adhere directly to endothelium when either the ECs and/or platelets are activated by agents such as thrombin or ADP (platelets) or the cytokines IL-1β, TNF-α or TGFβ1 (EC) [39, 41–54]. Figure 2 shows the routes by which platelets may facilitate the recruitment of leukocytes to the vessel wall that are discussed in this review.

Intravital experiments in mice showed platelet adhesion to mesenteric venules following treatment with calcium ionophore and to arteries at sites of atheroma formation [55–57]. In addition, stimulation of the murine cremaster muscle for 4 hours by topical application of TGF-β1 results in platelet deposition in post-capillary venules [45]. Platelet adhesion in these models was supported by bridging of platelet αIIb/β3 integrin to EC αvβ3 integrin by von Willebrand factor (VWF) [41, 42, 44, 49, 52, 55] with a possible contribution from P-selectin [58]. An interesting mechanism for delivering platelets to EC was reported by Van Gils et al. [59] who showed that platelets in aggregates with monocytes (a phenomenon discussed in detail below) relocated to the rear of the leukocyte before detaching and adhering to an EC upon monocyte diapedesis. One can speculate that this process might be relevant to the secondary recruitment of blood borne leukocytes to the vessel wall during inflammation.

Platelets adherent to the vessel wall can recruit blood borne leukocytes

The concept that platelet deposition to the vessel wall can promote the recruitment of leukocytes now has substantial experimental support. *In vitro*, numerous studies demonstrate platelet-mediated leukocyte recruitment to adhesive substrates of matrix proteins or ECs. Thus, when whole blood was perfused across sub-confluent cultures of ECs, platelets bound to exposed matrix and subsequently supported the recruitment and activation of perfused neutrophils [60]. In flow based models of vascular

damage, platelets deposited from whole blood onto collagen could recruit leukocytes [61, 62], and this was most efficient under conditions of disturbed flow in a model recapitulating the environment found downstream of atherosclerotic plaque. In this model, platelets and leukocytes were delivered to the substrate in a continuously rotating vortex which developed downstream of a step in the flow chamber [63]. Using whole blood under flow conditions, even platelets that were sparsely adherent to ECs could efficiently recruit neutrophils and monocytes [45, 61]. Interestingly, secondary recruitment of monocytes to ECs was supported by platelet-monocyte aggregates that were adherent to the endothelium, with platelet P-selectin acting as a bridge to deliver flowing monocytes to the EC [64]. Where it was characterised in these studies, authors showed that leukocyte capture was mediated by platelet P-selectin and stable adhesion was supported by leukocyte β2-integrins.

An important aspect of these studies is the diversity of leukocytes recruited by vessel wall adherent platelets. Thus, these as well as other reports [65, 66] detailed the platelet-mediated recruitment of neutrophils, monocytes, dendritic cells, T-lymphocytes, B-lymphocytes and NK-cells to endothelium. This lack of specificity is not surprising, as the counter ligand for P-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), is present on many leukocyte subsets. However, a number of observations describing specificity in the platelet-mediated recruitment of leukocytes are of particular interest. Kuckleberg et al. [45] demonstrated that monocytes were preferentially recruited by EC adherent platelets in an EC-smooth muscle cell co-culture model of the atherosclerotic artery wall. The ECs were activated by TGF-β1 generated in co-culture, and could recruit flowing platelets to surface VWF, a model that could be recapitulated by activating ECs with recombinant TGF-β1. Specificity for monocytes arose through EC production of CCL2 (MCP-1), which preferentially stabilised the adhesion of monocytes captured from flowing whole blood by platelet P-selectin. Two other groups using intravital observations in atherosclerotic mice (*ApoE−/−*) also observed chemokine dependent specificity of monocyte adhesion to the carotid artery [56, 67]. In these studies, adhesion of platelets which presented P-selectin, and deposition of platelet-derived CCL5 (Regulated on Activation, Normal T cell Expressed and Secreted – RANTES) and CXCL4 (platelet factor-4 – PF-4) onto the endothelium, was responsible for increased monocyte adhesion. Schulz et al. demonstrated a similar paradigm on cytokine stimulated human ECs [68]. Here, platelets binding from whole blood captured leukocytes through P-selectin and monocytes were subsequently activated by EC derived CX3CL-1 (fractalkine). In addition, blockade of CX3CR1 by injection of a function neutralising antibody into the *ApoE−/−* mouse, inhibited the adhesion of WEHI-274.1 cells (a monocytic cell line) to the carotid artery. Thus, although the initial tethering of leukocytes by platelet P-selectin may not be selective, specificity for recruitment of leukocyte subsets, in particular monocytes can be achieved by the presentation of chemokines, the receptors for which are restricted to a limited repertoire of leukocytes.

The formation of heterotypic leukocyte–platelet aggregates in the blood

The formation of platelet–leukocyte aggregates in the blood, and the subsequent presentation of platelet adhesion receptors on the leukocyte, would mean that circulating leukocytes would possess a means of adhesion to the vessel wall that is not normally present during inflammation. The formation of heterotypic platelet–leukocyte aggregates in blood is dependent upon platelet activation [69]. This leads to platelet P-selectin binding to leukocyte PSGL-1 to establish heterotypic adhesion [70]. Indeed, blocking the function of P-selectin or PSGL-1 with neutralising antibodies is an effective means of ablating aggregate formation [70–72]. Importantly, intercellular adhesion is further stabilised directly or indirectly (via fibrinogen bridging) by other receptors, e.g. GPIb and αIIbβ3 integrin on platelets and the β2 integrins (in particular, CD11b/CD18) on leukocytes [73, 74].

The formation of platelet–leukocyte aggregates occurs with a low incidence even in healthy individuals. Indeed, platelet–monocyte aggregates appear to be more common in healthy children than in healthy adults [75], indicating that their occurrence might change with age. Interestingly, there is a positive correlation between the number of circulating leukocyte–platelet aggregates and the severity of inflammatory and infectious diseases. Thus, an increase in aggregates is reported in diabetes and rheumatoid arthritis, diseases associated with accelerated formation of atheroma and increased risk of athero-thrombotic disease [76–78]. An increase in aggregates has also been observed in inflammatory bowel disease [79] and ischaemic heart failure [80]. Interestingly, their incidence is also increased in bacterial infections [81]. Indeed, monocytes with adherent platelets are recruited to sites of *Leishmania major* infection [81], while *Staphylococcus aureus* promotes the formation of neutrophil–platelet aggregates in the circulation [82].

The formation of platelet–leukocyte aggregates (in particular platelet-monocyte aggregates) occurs in atherosclerosis and athero-thrombotic disease. Thus, in both stable coronary artery disease or during coronary or cerebral infarction, the number of circulating aggregates increases significantly [83–89]. Moreover, the incidence of aggregates increases with independent risk factors for cardiovascular and cerebrovascular disease, such as hypertension [90, 91]. Importantly, increases in circulating platelet–monocyte aggregates may themselves be an independent risk factor for cardiovascular and cerebrovascular disease, or at least, their abundance can be used to stratify patients on the basis of predicted outcome after acute myocardial infarction [92]. Interestingly, experiments tracking autologous infused biotinylated platelets (activated *ex vivo*) in animal models demonstrate that the number of circulating monocyte–platelet aggregates may be a more sensitive indicator for *in vivo* platelet activation than circulating neutrophil-platelet aggregates or P-Selectin-positive non-aggregated platelets. Indeed, it has been demonstrated that after percutaneous coronary intervention (PCI), there is an increased number of circulating monocyte–platelet aggregates (and, to a lesser extent, neutrophil-platelet aggregates), but not P-selectin-positive platelets in peripheral blood. These data imply a rapid and efficient association between circulating leukocytes and activated platelets, making the later difficult to observe.

Thus, these studies indicate that heterotypic platelet–monocyte aggregates are associated with the progression of atherosclerotic disease and may contribute to the severity of symptomatic athero-thrombotic disease. However, it is notable that a direct role of heterotypic aggregate formation in the recruitment of monocytes (or other leukocytes) to ECs overlying atherosclerotic plaque has yet to be described.

The role of PMV in promoting leukocyte recruitment

Microvesicles are heterogeneous plasma membrane derived particles, 100–1000 nm in diameter, which can be detected in blood, urine and other bodily fluids [93]. They are released from cells of the vasculature, including platelets, ECs and leukocytes, and specific populations can be identified using appropriate methodology (e.g. flow cytometry), as they express surface markers derived from their cell of origin. There is mounting evidence that microvesicles play a physiological role in inflammation [94].

Microvesicles are released from activated cells and inflammatory diseases are associated with increased numbers of circulating microvesicles [95]. Microvesicles may be incorporated directly into the plasma membrane [96] or internalised by a recipient cell [97], thereby delivering vesicle contents. As microvesicles transport a wide range of molecular cargo, including DNA [98], mRNA [99], micro-RNA [100], membrane and signalling lipids [101] and proteins [102], there is the potential for important modification of recipient cell function. Importantly, this can include alterations in their adhesion to other cell types.

Microvesicles derived from activated platelets (PMV) are considered to be the most abundant population in human blood [103]. *In vitro*, PMV have been generated in response to shear stress, thrombin, calcium ionophore, ADP, collagen and collagen related peptide [104–107]. Interestingly, proteomic studies on populations of microvesicles derived using different platelet agonists demonstrate the formation of heterogeneous populations. Thus, using thrombin or shear stress as distinct routes to platelet activation, resulted in microvesicles that differed in abundance, as well as the identity of their protein cargos [107]. Importantly, megakaryocytes, which are the bone marrow progenitor cells from which platelets mature, also release microvesicles into the blood. This population of microvesicles shares surface markers such as CD41 (αIIb-integrin subunit) and CD42b (GPIb) with PMV [103]. Thus, studies that use these molecules to discriminate a platelet-derived population of microvesicles should be interpreted with caution, as it is possible that the predominant population of microvesicles expressing CD41 and CD42b are derived from megakaryocytes. Only upon activation do platelets release microvesicles bearing CD62P (P-selectin) which may discriminate the origin of these populations.

An increased incidence of circulating PMV is associated with a number of diseases. In diabetic retinopathy, the number of PMV was associated with the severity of disease [108], while the levels of PMV circulating in type-1 diabetics correlated with the degree of pro-atherogenic dyslipidaemia [109]. There was a correlation with vascular dysfunction (assessed by measuring arterial elasticity and flow-dependent vasodilatation of the brachial artery) [110] in patients with Type-2 diabetes. Interestingly, the number of PMV was higher in patients with acute coronary syndromes than those with stable angina [111], implying an association with the onset of athero-thrombotic disease.

The roles of PMV in inflammation and pathogenesis of inflammatory disease is not well understood. However, they possess adhesion receptors such as GPIb, αIIbβ3-integrin and P-selectin, meaning that they could interact with the vessel wall and circulating leukocytes to promote recruitment of the later. Surprisingly, their role in leukocyte adhesion is sparsely reported. Tersteeg et al. [112] showed that platelets adherent to physiological substrates *in vitro* and *in vivo* elaborated extensive (100's μm) flow-induced membrane protrusions (FLIPRs) which delivered microvesicles to neutrophils and monocytes rolling in a P-selectin dependent manner. These interactions lead to leukocyte activation using increased CD11b expression and the shedding of CD62L (L-selectin) as markers. The interaction of PMV with ECs has also been studied. Mause et al. [113] demonstrated that PMV rolled on IL-1β stimulated human microvascular ECs using P-selectin and GP1b. This was sufficient for the deposition of microvesicle derived CCL5 onto the ECs, leading to increased arrest of perfused monocytes [113]. Barry et al. [114, 115] observed that the adhesion of monocytes and the monocytic leukaemia cell line, THP-1, to ECs treated with PMV was increased, an outcome that was attributed to the activation of both ECs and leukocytes by the delivery of arachidonic acid (AA) from the microvesicles. Lindemann et al. showed that activated platelets could synthesise IL-1β which was incorporated into PMV [116]. Importantly, IL-1β-mediated activation of ECs by these microvesicles resulted in neutrophil adhesion, although the molecular basis of recruitment was undescribed. PMV also up-regulated adhesion receptors (including E-selectin and ICAM-1) on ECs [100]. PMV also interact directly with leukocytes. Consequently, the expression of adhesion receptors such as CD11b and CD32 and secretion of TNF-α, IL-1β and IL-6 were up-regulated in THP-1 cells [106]. Furthermore, P-selectin expressed on PMV acted as an adhesive bridge between HL-60 neutrophilic cells, leading to the formation of homotypic leukocyte aggregates in flow [117].

Thus, it is clear that PMV increase in the circulation during inflammatory disease. However, at present, it is unknown whether this is a cause or consequence of pathology. Nevertheless, PMV appear to have the potential to amplify inflammation by virtue of the interaction with both leukocytes and ECs.

The role of platelets in leukocyte recruitment in IR injury

Ischemia and reperfusion (IR) injury is a key contributor to the pathology of cardiovascular and cerebrovascular infarction (i.e. stroke and heart attack). It may also occur as a complication of reconstructive vascular surgery or organ transplantation. It is widely recognised that reperfusion of ischemic tissue exacerbates tissue damage by inducing a marked inflammatory response [118]. It is thus pertinent to consider the role of platelets in leukocyte recruitment, a process which is fundamental to the progression of IR injury [119–122].

Using electron microscopy and immuno-histochemistry, Cywes et al. [123, 124] demonstrated the involvement of platelets in IR injury. They noted platelet accumulation in rat and human livers subject to cold perfusion as a means of preservation prior to transplant (otherwise called preservation injury), a process that imposes an ischaemic insult on the tissue which is reperfused upon transplant. Since then, platelets have been implicated in the progression of IR injury during transplant of other organs including the heart [125], lung [126], liver [124, 127] and pancreas [128]. Ischemia and reperfusion leads to the activation and accumulation of platelets within vascular beds early after reperfusion [129], and platelets are among the first cells recruited to the site of injury [130]. Intravital microscopy has demonstrated that platelets interact with numerous vascular beds in the mouse liver (arterioles, sinusoidal capillaries and venules) after ischemia followed by as little as 20 minutes of reperfusion. The injury induced resulted in hepatocellular and microvascular injury and failure of sinusoidal perfusion [127, 131, 132]. Interestingly, the extent of platelet recruitment to the vascular endothelium is dependent upon the duration of ischemia, and not directly to the duration of reperfusion [127]. P-selectin plays an important role in the adhesion of platelets to the vessel wall during post-ischeamic reperfusion [58, 130, 131, 133–136]. However, the source of P-selectin which mediates these processes is unclear. Different studies indicate that EC [58, 130, 132, 134] or platelet-derived P-selectin [133] or both pools of the molecule [133] are involved in the recruitment of platelets to ECs. Khandoga et al. [131] described an interaction between fibrinogen deposited on ICAM-1 of post-ischaemic ECs, which resulted in platelet deposition in the liver [131], while a number of studies identified platelet αIIbβ3 as the counter ligand for fibrinogen deposited in arterioles and venules following ischemia of different tissues [133, 137–139]. Others have shown roles for reactive nitrogen species [140], angiotensin II type 1-receptors [AT(1)] [141], endothelin-A [142] and lymphocyte derived IFN-γ [143] in promoting the adhesion of platelets to the post-ischaemic vasculature.

There is also substantial evidence that platelets are responsible for a proportion of the leukocyte recruitment observed after

reperfusion of ischemic tissue. Thus, Lefer et al. [144] showed that platelets were important for neutrophil recruitment during reperfusion of isolated rat hearts that had been subject to ischaemia. A number of studies using intravital imaging of the vasculature after ischaemia and reperfusion showed that platelets were important for leukocyte recruitment in the microcirculation of the mesentery [145], the small intestine [146], in cerebral vessels, as well as in the retina [134]. Not surprisingly, they all reported a central role for P-selectin in the recruitment of leukocytes.

Platelet-mediated leukocyte adhesion has also been directly implicated in the process of reperfusion injury. Thus, platelets contributed to neutrophil-mediated cardiac dysfunction of isolated perfused rat hearts when mixtures of these cells were perfused after ischaemia [144]. Importantly, platelet depletion during IR preserves organ function in a number of models. Thus, the canine pancreas [128] and liver [147] benefited from such a strategy. Singbartl et al. [148] showed that platelet P-selectin was necessary for neutrophil-mediated acute ischemic renal failure in mice. In addition, in rats, the retinal microcirculation was spared if animals were rendered thrombocytopenic 4 hours prior to ischemia and reperfusion [134].

Thus, platelets are efficiently recruited during the reperfusion of ischemic tissue, and play a role in the recruitment of inflammatory leukocytes, which in turn are responsible for tissue damage. Moreover, blockade of platelet adhesion to post-ischemic vessels, their removal from the circulation prior to ischaemia, or the inhibition of platelet borne P-selectin, can significantly attenuate the severity of reperfusion injury. However, other studies have shown protective effects of platelet-neutrophil aggregate in resolution of inflammatory responses in the microcirculation during IR [149]. Indeed, it appears that platelet–neutrophil interactions allow release of the pro-resolving lipid, lipoxin A4 via fpr2/3, which controls formation of platelet–neutrophil aggregates. Similarly, production of maresin-1, a pro-resolving mediator, also depends upon interaction of neutrophils with platelets in a model of acute lung injury [150]. Indeed, the process of trans-metabolic production of pro-resolving mediators by platelet–leukocyte aggregates in inflammation is a burgeoning field, and the interested reader is directed to several excellent reviews of this area.

Downstream consequences of platelet–leukocyte interactions for leukocyte function

The consequences of platelet binding for leukocyte function and fate have not been extensively investigated. However, acute modifications to leukocyte surface receptors are documented. Thus, in heterotypic aggregates, monocyte L-selectin is shed [151], while the expression and activity of β1- and β2-integrins is increased [65, 151–154]. Changes in the markers that define monocyte subsets are also altered by platelet binding. Thus, in a PGE2 driven process, CD16 on monocytes was increased in heterotypic aggregates formed *in vivo* after influenza vaccination or *in vitro* by mixing monocytes with platelets [155].

Leukocyte function after adhesion and activation on monolayers of activated platelets also changes. Thus, neutrophils activated on this substrate by IL-8, fMLP or C5a, released more neutrophil elastase than neutrophils in suspension. This process required β2-integrin (CD11b/CD18)-mediated binding to the platelets, and the production of LTB4 [33]. Neutrophils also receive signals from platelet adhesion receptors (P-selectin and CD31) which regulate the direction [32] and the velocity [31] of their migration. Platelet monolayers also supported the migration of adherent neutrophils into large, homotypic aggregates which were prone to detachment and dissemination downstream by flow, in a model of embolus formation [156]. The influence of platelet adhesion and/or exposure to platelet-derived agents such as chemokines has been reported to influence the function of a number of lymphocyte subsets, including helper T-cells, cytotoxic T-cells, regulatory T-cells, B-cells and NK cells. This area of lymphocyte biology has recently been reviewed and the reader is directed to the manuscript by Li et al. [65].

An aspect of leukocyte biology that has received surprisingly little attention is the role of platelet adhesion in the fate of cells as they differentiate. Scheuerer et al. [157] demonstrated that the platelet-derived chemokine, CXCL4, promoted the survival and differentiation of monocytes into macrophages. Interestingly, monocytes maturing in the presence of oxidised-low density lipoprotein (Ox-LDL) into foam cells also showed a CXCL4-dependent increase in esterification and storage of cholesterol [158]. Using microarray analysis, Gleissner et al. [159] showed acquisition of a 'unique transcriptome' in human monocyte-derived macrophages when they were differentiated in the presence of CXCL4. Interestingly, human macrophages differentiated in the presence of CXCL4 were unable to up-regulate athero-protective genes such as heam oxygenase-1, due to down regulation of the haemoglobin scavenger receptor, CD16 [3, 160]. In addition, binding of platelets to monocytes via P-selectin induced the secretion of TNF-α, IL-1β, IL-6, IL-12, CXCL8 and CCL4 (MIP-1β) [161]. Seta et al. [162] showed that in the presence of platelets or platelet-derived supernatants containing CXCL12 (SDF-1), monocytes could differentiate into monocyte-derived multipotential cells (MOMCs). These authors had previously described MOMCs as 'primitive' cells with the potential to differentiate into mesenchymal and endothelial lineages [163].

The interaction between platelets and leukocytes as a therapeutic target in atherosclerosis

As the preceding discussion has demonstrated, platelets can play a role in the recruitment of leukocytes during inflammation, and this pathway probably contributes to pathogenesis in atherosclerotic disease [69, 164]. Indeed, platelets increase the selective recruitment of monocytes to endothelium in animal and human models of vascular inflammation [45, 151, 164, 165]. In view of this and the positive association between platelets–leukocyte interaction and the progression of atherosclerosis, targeting these pathways represents a therapeutic avenue of potential importance. Figure 3 shows the pathways that are amenable to therapeutic intervention using currently licensed agents or drugs under development.

The anti-platelet agents currently used in prevention of athero-thrombotic disease target platelet activation in thrombotic events [166–168]. While the anti-thrombotic efficacy of guideline-approved agents such aspirin and P2Y12 receptor antagonists (clopidogrel, prasugrel and ticagrelor) is well established, recent evidence suggests they may have additional anti-inflammatory effects in addition to being platelet activation inhibitors [166–168]. Thus, Aspirin inhibits platelet activation by irreversibly binding to the platelet cyclooxygenase (COX)-1 enzyme, abolishing the formation of thromboxane (Tx)A2 [169]. Aspirin is ≈150 times more potent at acetylating ser529 on COX-1 than ser516 on its inducible homologue COX-2 [169]. Thus, at doses used for prevention of athero-thrombotic disease, aspirin has a negligible anti-inflammatory effect [166]. It is perhaps not surprising therefore that these doses of aspirin do not affect platelet or leukocyte activation and aggregate formation in whole blood [170].

Production of ADP by activated platelets amplifies platelet responses to activation, e.g. by stabilising platelet aggregation. ADP acts through the P2Y12 receptor [171]. Thus, the addition of a P2Y12 antagonist to aspirin offers better protection against athero-thrombotic ischemic events, especially in patients suffering from acute coronary syndromes or having undergone percutaneous coronary intervention [167]. There are currently three anti-platelet

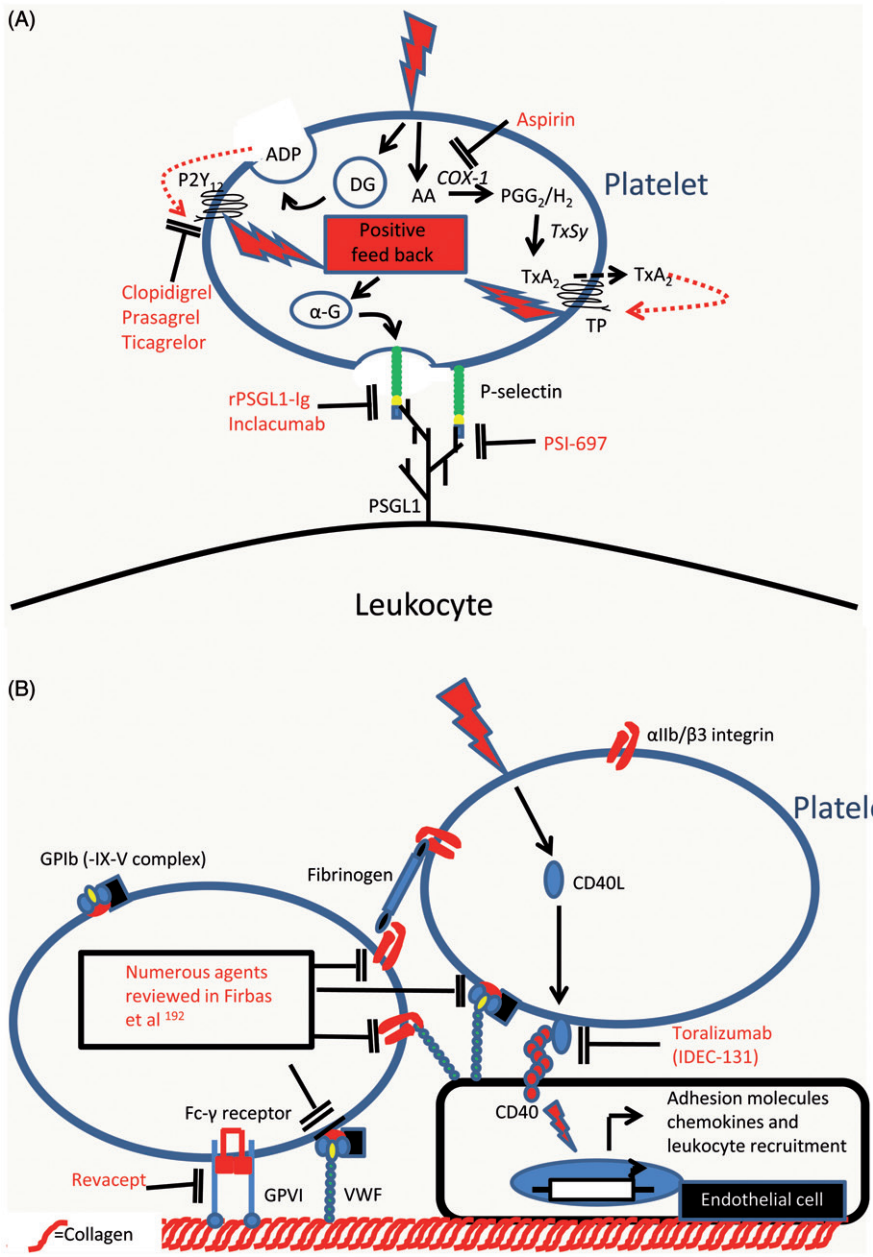

Figure 3. Targets for therapeutic intervention in platelet–leukocyte adhesion and in the platelet-mediated recruitment of leukocytes: (A) Therapeutic agents that inhibit the adhesive interactions between platelets and leukocytes or which inhibit the activation of platelets by antagonising platelet derived positive feedback loops which amplify the platelet response to primary activating stimuli. (B) Therapeutic agents that inhibit platelet adhesion to the vessel wall by either antagonising adhesive pathways directly or by inhibiting endothelial cell activation in response to platelet borne activating stimuli. AA, Arachidonic acid; ADP, Adenosine diphosphate; α-G, Alpha granule; COX-1, Cyclooxygenase-1; DG, Dense granule; P2Y12, ADP receptor; PGG2, Prostaglandin G2; PSGL1, P-selectin glycoprotein ligand1; TP, Thromboxane A2 receptor; TxA2, Thromboxane A2; TxSy, Thromboxane synthase.

agents targeting this receptor: clopidogrel, and the newer generation prasugrel and ticagrelor [171]. In addition to potent antithrombotic efficacy, treatment with *clopidogrel* has been associated with a number of anti-inflammatory effects. In patients with atherosclerosis, clopidogrel caused a reduction in circulating CD40L and CCL-5, improved endothelial nitric oxide bioavailability, reduced platelet P-selectin expression and impaired formation platelet–leukocyte aggregates [172–177]. Interestingly, Antonino et al. showed that inhibition of inflammation markers correlated with inhibition of platelet activation, thus strengthening the hypothesis that clopidogrel's anti-inflammatory effects are driven by inhibition of platelet activation [178]. *Prasugrel* is a P2Y12 receptor antagonist with more uniform and potent platelet inhibition than clopidogrel [171]. Consistent with this, prasugrel showed a greater reduction in heterotypic platelet–monocyte aggregates and levels of circulating CD40L [179–181]. Unlike clopidogrel and prasugrel, which require bioactivation by the liver, *ticagrelor* is a direct-acting reversible P2Y12 receptor antagonist [171]. In trials, ticagrelor was superior to clopidogrel in antithrombotic effects and in reduction of clinical endpoints of cardiovascular events and death [182]. Despite this, no differences in inflammatory markers was seen between clopidogrel- and ticagrelor-treated patients [183]. These studies imply that P2Y12 receptor antagonists have additional anti-inflammatory properties which go beyond inhibition of platelet activation.

Despite the anti-inflammatory effects seen with current antiplatelet agents, their use in secondary prevention of cardiovascular disease does not prevent athero-progression in humans [184]. This may be because their use is initiated late in disease development, often after an athero-thrombotic event. Alternatively, we may require a different approach to target this aspect of disease with greater precision, of which, inhibiting platelet-mediated leukocyte recruitment could be an important therapeutic endpoint. As platelet-facilitated leukocyte recruitment requires P-selectin interactions with PSGL-1 [185], inhibitors of this thrombo-inflammatory axis are in development. Among the earliest, a recombinant antagonist (rPSGL-Ig) was promising in pre-clinical studies. It significantly reduced the formation of heterotypic aggregates, attenuated infarct size, protected against IR injury and accelerated thrombolysis in animal models [186–190]. However, in clinical trials, the molecule showed no benefit to coronary vessel patency, infarct size or reperfusion and

functional recovery of the myocardium. Indeed, clinical outcomes were unaltered at 1 or 6 months [191, 192].Consequently, development was stopped and ongoing studies terminated. Notwithstanding, novel P-selectin inhibitors are in development. A small molecule P-selectin antagonist, PSI-697, was shown to reduce *ex vivo* thrombus formation in humans, but failed to inhibit formation of heterotypic aggregates *in vivo* [71, 193]. Inclacumab, a human anti-P-selectin antibody, inhibited the formation of heterotypic aggregates in healthy volunteers and in patients with elevated numbers of circulating aggregates [72]. Whether these molecules will confer clinical benefit remains to be seen.

The CD40–CD40L interaction is an important link between platelet activation and inflammation [194]. Binding of platelet-released CD40L to CD40 on ECs, monocytes/macrophages, B cells and smooth muscle cells induces activatory changes that result in a pro-inflammatory phenotype [194]. Attempts have been made to block this interaction in order to halt athero-progression. In animal models, infusion of anti-CD40L antibodies delayed lesion formation, reduced leukocyte recruitment to the vessel wall and inhibited athero-progression [195–198]. A humanized antibody against CD40L (toralizumab, IDEC-131) was taken to phase II clinical trials in diseases with a strong inflammatory component (namely, multiple sclerosis and Crohn's disease). Toralizumab's development was stopped when a strong signal for unexplained risk of thrombosis emerged [199]. While the reason for thrombotic risk remains unexplained, development of compounds targeting the CD40L signalling pathway continues [194].

Intervention in platelet-mediated recruitment of leukocytes may also halt athero-progression. Platelet activation through the binding of the GPIb receptor to VWF contributes to leukocyte recruitment at high shear [200, 201, see above]. Moreover, blockade of platelet adhesion to arterial endothelium in atherosclerotic mice reduces the development of plaques as well as leukocyte recruitment to lesions [57 and see above]. Numerous inhibitors, antibodies, peptides and small molecules have been developed to target this interaction with promising pre-clinical results. As the prospects for therapies based on targeting this interaction have recently been discussed, the reader is directed to the review by Firbas et al. for further information [202]. Platelet adhesion at sites of vascular injury is an orchestrated event, in which the collagen receptor GPVI plays an important role [203]. Inhibition of GPVI protected animals from developing atherosclerotic lesions in mouse and rabbit models [204]. Revacept, a dimeric GPVI-Fc, favourably modified plaque morphology in atherosclerotic rabbits [205] and reduced cerebral infarct size and oedema in murine stroke [206]. This agent also inhibited collagen-induced responses in healthy volunteers, and was safe in a phase I trial [207]. Whether this molecule will have an impact on athero-progression in humans again remains unknown.

Conclusions and future perspectives

There is now strong evidence that platelet–leukocyte interactions within the vasculature increase in cardiovascular disease, or even upon acquisition of risk factors for such diseases (e.g. hypertension or dyslipidaemia). A major question to be answered is whether these interactions are consequential or causal in inflammatory disease. Certainly, platelets are important in disease progression in animal models of atherosclerosis. Moreover, both *in vivo* and *in vitro* data suggests that part of their contribution to pathology is by recruitment of leukocytes to the vessel wall. Apparently direct interaction of platelets with arterial ECs can lead to this increase in leukocyte trafficking. Interestingly, there is sufficient evidence to suggest that this process selectivity recruits monocytes, which are the precursors of inflammatory foam cells in atherosclerotic plaque. Other modes of platelet leukocyte interaction that lead to leukocyte recruitment should not be discounted. The formation of heterotypic aggregates in circulating blood is known to increase in many pathologies, including cardiovascular diseases. However, whether these interactions are consequential or causal of disease remains to be determined experimentally. The developing field of microvesicle biology is likely to have an important influence on our understanding of platelet-mediated leukocyte recruitment in cardiovascular diseases. PMV play an important role in the thrombotic disease and coagulopathies [208] and they have been shown to interact with ECs *in vitro*, resulting in the recruitment of leukocytes. Like platelets, PMV form heterotypic aggregates with leukocytes in whole blood (unpublished observations). Again, whether these interactions are consequential or causal of disease remains to be determined experimentally.

So far, the known routes of platelet-mediated leukocyte recruitment are based on adhesion receptors and signalling pathways that regulate haemostasis and leukocyte trafficking during inflammation. However, it would be naïve to think that these represent an exhaustive list. Probably, new adhesive and regulatory pathways are still to be described. Indeed, the identification of CLEC-2 [209], a novel activation receptor on platelets is an exemplar of such a scenario. The only known physiological ligand for CLEC-2 is podoplanin [210], and Herzog et al. [211] showed that interactions between platelet borne CLEC-2 and podoplanin on the fibroblastic reticular cells cuffing high endothelial venules (HEV) of lymph nodes was necessary for integrity of this vascular bed and to ensure the regulated traffic of lymphocytes. Although this interaction maintains a homeostatic pathway in the immune system, it is likely that novel modes of platelet–leukocyte–vessel wall interaction will support pathological interactions in cardiovascular disease.

Whether intervening in platelet-mediated leukocyte recruitment will have efficacy in cardiovascular disease is still an open question. In animal models, blockade of platelet activation or platelet adhesion retards plaque development. However, in patients with established atherosclerosis, current anti-platelet therapies do not effect disease progression. Current therapeutic options were not designed to target platelets involved in these pathways and this could account for this lack of efficacy. As our understanding of the biology underlying these pathways increases, so should our ability to precisely target them with pharmacological agents.

Declaration of interest

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